the reduction in surface charge density on the cell after sialidase treatment enhances phagocytosis. The role of the spleen in sequestration of erythrocytes has been assessed by GARDNER et al.[39] and by OWEN et al.[47].

It has also been shown[48,49] that macrocytic erythrocytes disappear from the circulation at a faster rate than normocytic erythrocytes and this high susceptibility to destruction might be due to a physical defect and metabolic anomaly as well as to a low surface charge.

In conclusion it can be envisaged that erythrocytes at different degrees of maturity have a different surface charge which is regulated by the cell internal metabolism and possibly by the action of plasma or tissue sialidase. A low surface charge and increased tendency to adhere to reticuloendothelial cells may be the condition that determines the removal of a large part of macrocytes[49] and old erythrocytes from the circulation[50].

Riassunto. Le proprietà della membrana dell'eritrocita variano in rapporto all'età della cellula e probabilmente all'azione della sialidasi. Una diminuzione della carica elettrica dell'eritrocita appare essere la condizione determinante l'eritrofagocitosi.

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STUDIORUM PROGRESSUS

The Effect of Insulin on the Glycogen Metabolism of Isolated Fat Cells

LEONARDS and LANDAU[1] showed that insulin plus low concentrations of glucose stimulated the incorporation of glucose C-1 and C-6 into glycogen and fatty acids, and the oxidation of glucose C-1 to carbon dioxide by epididymal adipose tissue in vitro. High concentrations of glucose favoured the oxidation of glucose C-6 to carbon dioxide and the incorporation of glucose carbon into the glycerol moiety of triglycerides.

In the presence of insulin and (U^{14}C)glucose the rat epididymal adipose tissue and the mouse hemidiaphragm in vitro synthesize more glycogen than can be accounted for by incorporation of exogenous glucose into glycogen[2]. In the absence of glucose, insulin can stimulate the synthesis of glycogen by the mouse hemidiaphragm in vitro[2].

These data support the hypothesis that insulin has a direct effect on the metabolism of glycogen in certain tissues in vitro. This paper reports the effects of different concentrations of insulin on the conversion of (1^{14}C)glucose to (^{14}C)glycogen, (^{14}C)triglycerides and (^{14}C)O_2 by isolated fat cells.

Materials and methods. Male Wistar rats 110–130 g (Novo Terapeutisk Laboratorium A/S, Copenhagen) were allowed food and water ad libitum until used. Human serum albumin (Swiss Red Cross Blutspendedienst, Bern, Switzerland) was purified by dialysis[3]. Ten times crystallized bovine insulin (24.4 U/mg, lot No. 0818864) was obtained from the Novo Research Institute. Unless otherwise stated, the chemicals used throughout this work were of analytical grade (Merck AG). (1^{14}C)glucose (2.96 mC/mmole) was purchased from The Radiochemical Centre, Amersham, England. Collagenase was bought from the Sigma Company, USA.

Free fat cells were prepared by disruption of rat epididymal adipose tissue with collagenase (RODBELL[4], GLIEMANN[5]). The suspension was diluted to about 10^5 cells/ml in bicarbonate buffer (KREBS and HENSELEIT[6]), pH 7.4, which contained 10 mg of albumin/ml and 0.55 m*M* glucose. The concentration of cells in the suspension was measured[5]. In some experiments, the triglycerides were extracted from aliquots of the cell suspension, dried and weighed[5]. Standard concentrations of insulin were prepared according to GLIEMANN[5].

Twelve flasks were prepared with insulin-free buffer, and 6 with each of the insulin standards. (1^{14}C)glucose was added to the cell suspension to a final specific activity of 70–80 nC/mmole of glucose. 1 ml of the cell suspension was then pipetted into each incubation flask. The flasks were gassed with 95% O_2/5% CO_2 and stoppered. Immediately after the addition of the cell suspension, the contents of 6 of the insulin-free flasks were analysed. The ^{14}C recovered in each metabolite from these flasks – the cell blanks (CB) – served as the background for the determination of ^{14}C. The remaining flasks were incubated for 2 h at 37 °C, with shaking (50 strokes/min). Three flasks from each group were used for the isolation of (^{14}C)O_2 and the (^{14}C)triglycerides. The remaining 3 flasks were used for the preparation of the (^{14}C)glycogen. The concentration of glucose in the medium was determined by the glucose oxidase method[7], and the ^{14}C by liquid scintillation counting[5]. The (^{14}C)O_2 and (^{14}C)triglycerides were recovered and determined according to GLIEMANN[5].

After the incubation, the cell suspension was filtered through Oxoid filters (22–24 mm diameter, pore size 0.45 μ). The flasks were washed once with bicarbonate buffer[6] containing 10 mg albumin/ml and once with albumin-free bicarbonate buffer, and the washings were transferred to the filters. The filters were then washed with 2 ml of triglyceride extraction medium[8]. Each filter was transferred to 3 ml of KOH (30 g/100 ml containing 100 μg of glycogen/ml) in a polypropylene centrifuge tube.

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The glycogen was extracted from the cells by heating the tubes at 100 °C for 30 min. The glycogen was precipitated by the addition of 25 μl of Na_2SO_4 (sat.) and 6 ml of absolute alcohol/tube[9]. The tubes were kept at — 20 °C for 30 min, and then centrifuged for 35 min at 4000 *g* at 4 °C. The supernatants were removed, the glycogen suspended in 2 ml of 66% alcohol, and the samples recentrifuged. The glycogen was dissolved in 2 ml of water and transferred to a scintillation vial with 20 ml of scintillator[10].

The radioactivity in 20 glycogen fractions was followed through 3 successive precipitations. Samples of cell glycogen were also converted to glucose by acidic and enzymatic hydrolysis[11]. The free glucose was converted to the osazones[12]. The osazones were burnt to CO_2[13], and the resultant (^{14}C)O_2 measured.

All ^{14}C determinations were carried out in Packard Tri-carb liquid scintillation counters. All samples were counted until at least 2000 counts had been registered. The different counting systems were coordinated by counting small amounts of (1^{14}C)toluene in each system. Quench was estimated by using internal standards of (1^{14}C)naphthalene. The (^{14}C)glycogen determinations were corrected for the observed quenching (less than 2%).

The specific activity of the glucose in the medium was calculated from the amount of glucose and the amount of radioactivity/ml of medium.

The crude cpm in each fraction were converted to *n* atoms of (^{14}C) incorporated into each metabolite/10^5 cells/2 h incubation.

Statistical methods. Each separate experiment was designed to test: (1) the significance of the effects of insulin on the incorporation of glucose into the different metabolites; (2) the significance of the differences between the amounts of glucose incorporated into each metabolite at the given levels of insulin; (3) the significance of the differences between the responses of the different metabolites to changes in insulin level.

Standard analyses of variance (HALD[14]) were performed on the logarithms of results from the experiments. The homogeneity of the variances was evaluated by means of BARTTLET's test (HALD[15]), and found to be acceptable after the logarithmic transformation. Similar analyses of variance were performed on the logarithms of the ratios between the corresponding responses in 2 parameters.

Results. The ^{14}C in the glycogen fraction was measured after 1 precipitation from KOH and after 2 further precipitations. Only the cell blanks showed a significant ($P \leq 0.05$) loss of ^{14}C on reprecipitation. The mean conversions of (U^{14}C)glycogen from 4 groups of cells to $^{14}CO_2$ (via the formation of glucose and glucosazones) were 83.8 $\pm$ 17% and 96.6 $\pm$ 15% after acid and enzymatic hydrolyses, respectively.

The effects of different concentrations of insulin on the conversion of (1^{14}C)glucose to (^{14}C)glycogen, (^{14}C)O_2 and (^{14}C)triglycerides were studied in 5 experiments. The results of one such experiment are shown in Figure 1. An increase in the concentration of insulin caused an increase in the conversion of glucose to these metabolites.

The 3 parameters differed in their responses to insulin. The conversion of (1^{14}C)glucose to (^{14}C)glycogen was more sensitive to insulin than the conversion of (1^{14}C)glucose to (^{14}C)O_2 or (^{14}C)triglycerides. The maximum effect of insulin on the conversion of (1^{14}C)glucose to (^{14}C)glycogen occurred at 10 μU of insulin/ml, whereas the maximum effect of insulin on the conversion of (1^{14}C)glucose to (^{14}C)O_2 and (^{14}C)triglycerides occurred at 1000 μU of insulin/ml. The analysis of variance of these data showed that there was a significant ($P = 0.0005$) interaction between the parameters and the insulin levels, i.e. different levels of insulin had different effects on the distribution of glucose within the metabolites.

The data from the 5 experiments were pooled, and an analysis of variance carried out on the combined results. The experiment-to-experiment variation, the effect of insulin on the incorporation of (1^{14}C)glucose into the different metabolites, and the interaction between the parameters and the insulin levels were all significant ($P = 0.0005$). When the experiment-to-experiment variance was combined with the between-replicates variance, to give an overall error variance, the difference between parameters, the effect of insulin on the parameters, and the interaction 'insulin level-parameters' were significant at $P = 0.0005$. The source of the interaction between the insulin levels and the parameters was located by determining the effects of different insulin levels on:

$$\log \frac{(^{14}C)O_2}{(^{14}C)\text{triglycerides}}; \quad \log \frac{(^{14}C)O_2}{(^{14}C)\text{glycogen}} \text{ and}$$

$$\log \frac{(^{14}C)\text{triglycerides}}{(^{14}C)\text{glycogen}}.$$

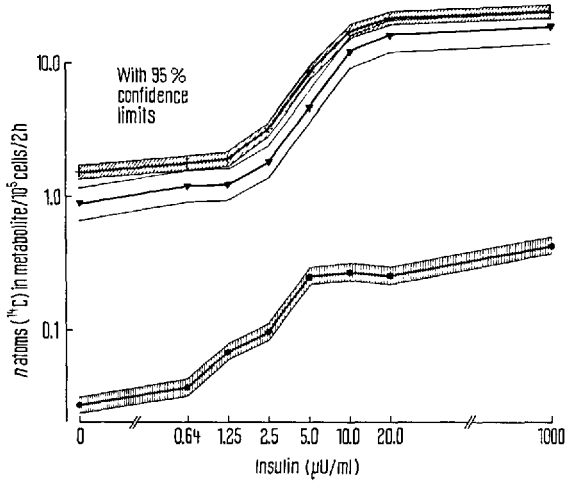

Fig. 1. The effect of different concentrations of insulin on the conversion of (1^{14}C)glucose to (^{14}C)glycogen (●——●), (^{14}C)triglycerides (▼——▼) and (^{14}C)O_2 (+——+) by isolated fat cells. Six samples of isolated fat cells were incubated for 2 h with (1^{14}C)-glucose (0.55 m*M*) and each of the concentrations of insulin shown in the Figure. The (^{14}C) content of each fraction was then determined as described in the text. The results are expressed as log *n* atoms (^{14}C) in each fraction/10^5 cells/2 h incubation (the mean triglyceride content of 7 different preparations of fat cells was 5.3 $\pm$ 1.1 ng/10^6 cells). The mean response of 3 samples and the 95% confidence limits are shown.

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An analysis of variance showed that there was no interaction between

$$\log \frac{(^{14}C)O_2}{(^{14}C)\text{triglycerides})}$$

and insulin levels between zero and 1000 μU/ml.

There was a significant interaction between insulin levels and the ratios:

$$\log \frac{(^{14}C)O_2}{(^{14}C)\text{glycogen}} \quad \text{and} \quad \log \frac{(^{14}C)\text{triglycerides}}{(^{14}C)\text{glycogen}}.$$

There was a significant ($P = 0.0005$) experiment-to-experiment variation in these ratios.

Figure 2 shows how

$$\log \frac{(^{14}C)O_2}{(^{14}C)\text{glycogen}}$$

was affected by insulin concentrations between zero and 1000 μU/ml. An increase in the concentration of insulin from 0–1.25 μU/ml caused an increase in the conversion of ($1^{14}C$)glucose to (^{14}C)glycogen, relative to its conversion to (^{14}C)O_2. This effect was reversed at higher concentrations of insulin.

Discussion and conclusions. An increase in insulin concentration (in the range of 0.16–20.0 μU/ml) increased the conversion of ($1^{14}C$)glucose to (^{14}C)O_2 and (^{14}C)triglycerides. These effects of insulin are qualitatively similar to those reported previously (RODBELL[4], GLIEMANN[3,5]). The stimulatory effect of insulin on the conversion of ($1^{14}C$)glucose to (^{14}C)glycogen was very much greater than that demonstrated by AUTOR et al.[16]. This difference may be a consequence of the techniques used, or of the glucose concentration in the incubation medium.

Within the ranges of insulin concentrations used, there was no significant effect of insulin on the conversion of ($1^{14}C$)glucose to (^{14}C)O_2, relative to its conversion to (^{14}C)triglycerides.

The conversion of ($1^{14}C$)glucose to (^{14}C)glycogen, relative to its conversion to (^{14}C)O_2 and (^{14}C)triglycerides, depended significantly ($P < 0.0005$) on the concentration of insulin. Between 0.16 μU and 2.5 μU of insulin/ml, the relative conversion of ($1^{14}C$)glucose to glycogen was higher than between 5.0 μU and 1000 μU of insulin/ml. It is concluded that extremely small concentrations of insulin cause a preferential utilization of glucose via the pathways of glycogen metabolism. The data presented here do not permit an unequivocal conclusion as to the mechanism of this effect.

The results may be interpreted in 2 ways. The enzymes of glycogen synthesis in the fat cell may have a high affinity for glucose-1-phosphate compared with the affinity of other pathways for glucose-6-phosphate. A small increase in the entry of glucose into the cell, caused by the increased transport of glucose in the presence of insulin, would then cause a preferential incorporation of glucose into glycogen. The synthesis of glycogen is very small compared to other pathways of glucose metabolism in the adipose tissue fat cell. The conversion of glucose to glycogen would become negligible as soon as the rate of entry of glucose into the cell exceeded the capacity of the glycogen synthesizing system, e.g. in the presence of high concentrations of insulin.

Alternatively, insulin may have a direct effect on the metabolism of glycogen in the fat cell. This explanation is supported by the experiments with epididymal adipose tissue previously cited[1,2,17].

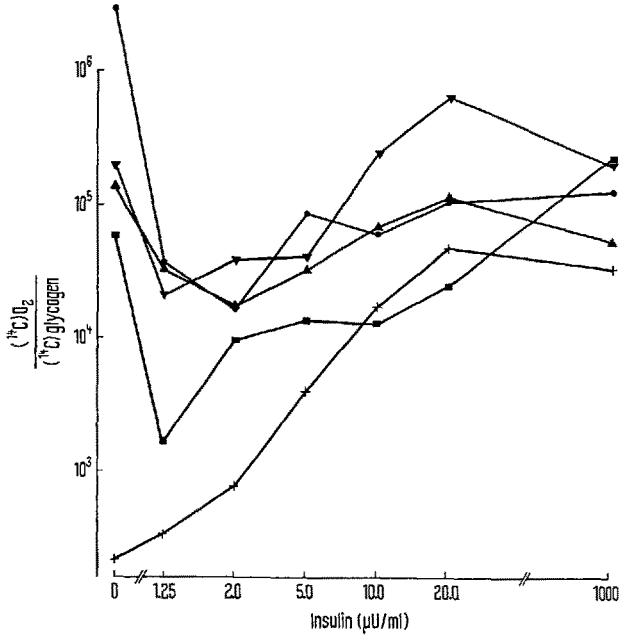

Fig. 2. The effect of insulin on the relative incorporation of ($1^{14}C$)-glucose into glycogen and CO_2 by isolated fat cells. The log of (^{14}C)O_2 over (^{14}C)glycogen was obtained from 5 experiments. The mean values for this ratio at each concentration of insulin are plotted against the concentration of insulin (on a log scale) for each experiment.

Résumé. (1) Des adipocytes isolés à partir du tissu adipeux épididymaire du rat ont été incubés dans des milieux contenant du glucose ($1^{14}C$) et des concentrations variables d'insuline. La quantité de glucose ($1^{14}C$) transformée en glycogène (^{14}C), (^{14}C)O_2 et triglycerides (^{14}C) a été déterminée. (2) On démontre que la quantité de glucose ($1^{14}C$) transformée en glycogène par des adipocytes peut être évaluée et qu'elle est fonction de la concentration d'insuline. (3) La comparaison entre les effets d'insuline sur les transformations du glucose ($1^{14}C$) en glycogène (^{14}C), en triglycerides (^{14}C) ou (^{14}C)O_2 révèle que l'insuline a une action apparemment directe sur l'incorporation du glucose au glycogène. (4) Ce fait serait donc dû à un effet direct de l'insuline sur une réaction dans la synthèse du glycogène par les adipocytes ou à une grande affinité des enzymes participant à la synthèse du glycogène pour le glucose-1-phosphate et à leur faible capacité pour la synthèse du glycogène.

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Novo Research Institute and Institute of Medical Physiology A of the University, Copenhagen (Denmark), 20 November 1967.

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